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ORIGINAL ARTICLE

Pepsin and pH of Gastric Juice in Patients With Gastrointestinal Reflux Disease and Subgroups

Pelin Ergun,*† *Sezgi Kipcak*,†‡ *Peter W. Dettmar, PhD*,§ *Jeanine Fisher*,§ *Andrew D. Woodcock*,§ *and Serhat Bor*†

Goal: The aim of this study was to investigate the pepsin values and pH results of gastric juice among the subtypes of gastroesophageal reflux disease (GERD) and functional heartburn.

Background: The major destructive agents of GERD on the esophageal epithelium are gastric acid and pepsin. No precise information about pepsin concentration in gastric juice exists.

Study: Ninety patients with GERD, 39 erosive reflux disease (ERD) LA grade A/B, 13 ERD LA grade C/D, 19 nonerosive reflux disease (NERD), 8 esophageal hypersensitivity, 11 functional heartburn, and 24 healthy controls were included in the study. During endoscopy gastric juices from the patients were aspirated and their pH readings immediately recorded. Gastric juice samples were analyzed using Peptest, a lateral flow device containing 2 unique human monoclonal antibodies to detect any pepsin present in the gastric juice sample.

Results: The highest mean gastric pepsin concentration (0.865 mg/mL) and the lowest median gastric pH (1.4) was observed in the LA grade C/D group compared with the lowest mean gastric pepsin concentration (0.576 mg/mL) and the highest median gastric pH (2.5) seen in the NERD group. Comparing pH, the NERD patient group was significantly higher ($P = 0.0018$ to $P = 0.0233$) when compared with all other GERD patient groups.

Conclusions: The basal gastric pepsin level in the healthy control group was comparable to literature values. There was good correlation and a significant linear relationship between the gastric pepsin level and gastric pH within the patient groups. The severity of the GERD disease is related to the lowest pH and the highest pepsin concentration in gastric juice.

Key Words: gastric juice, GERD, pH, pepsin, Peptest

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The study approval number 16-8.1/17, document number 70198063-050.06.04 was provided by the Ege University Clinical Research Ethics Committee, Izmir, Turkey. Informed consent was obtained from all individual participants included in this study. All participant data were anonymized before the final analysis of data.

S.B., P.W.D., and P.E.: contributed to the concept and design. P.E., S.K., and J.F.: gave the administrative support. S.B., P.E., and S.K.: contributed for provision of study materials. P.E., J.F., and A.D.W.: contributed to collection and assembly of data. A.D.W., J.F., and P.E.: contributed to data analysis and interpretations. P.W.D. and J.F.: contributed in manuscript writing. All authors: final approval of the manuscript.

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Gastroesophageal reflux disease (GERD) is responsible for causing damage to the upper gastrointestinal tract resulting in symptoms such as heartburn and regurgitation.[1] GERD can be broken down into phenotypes such as erosive reflux disease (ERD), nonerosive reflux disease (NERD), and esophageal hypersensitivity (EH).[2–4] NERD represents 70% of the GERD patients.[5] Functional heartburn (FH) is a condition that is not within the GERD spectrum but displays heartburn.[6] Subtypes of reflux diseases are usually caused by the esophageal defences being overwhelmed by stomach contents including acid and the digestive enzyme, pepsin. If the lower esophageal sphincter (LES) is compromised then it will lead to the flow of gastric juices into and above the esophagus.[7] Both conditions display typical symptoms of GERD, with studies also showing prevalence of atypical symptoms of GERD including sore throat and persistent cough[3]; treatment pathways are similar which include acid-suppression therapy such as proton pump inhibitors (PPIs),[8,9] although Chen and colleagues stated lower effectiveness in the NERD group.

EH is a condition in which the patient experiences a lower symptom threshold; therefore, patients report a more intense perception of symptoms such as heartburn and chest pain compared with individuals without hypersensitivity of the esophagus.[10] The causes of EH can be difficult to ascertain but studies have found previous acid reflux events that lead to sensitization.[10] Also individuals with EH symptoms report high levels of anxiety,[11] generally seen in women with a high association with irritable bowel syndrome.[4] Successful treatment can be limited because of the difficulty in pinpointing a cause.

FH is defined, based on Rome IV criteria, as typical heartburn symptoms in the presence of normal upper endoscopy findings (including normal biopsies), normal intraesophageal 24-hour pH monitoring, and a negative association between symptoms and reflux events.[12] Mechanisms that are responsible for the non-GERD-related condition are poorly understood. Giacchino et al,[2] Fass,[6] and Bilgi et al[11] report higher levels of stress and anxiety in FH patients. EH also plays a part in the causation of the condition, with the onset mechanisms correlated to peripheral or central sensitization.[13] Treatment in the first instance is commonly PPIs but in this clinical group the acid-suppressant drugs can prove to be noneffective FH.[2] Those patients whose symptoms can be contributed to psychological reasons may benefit from psychological therapy such as sessions with a psychiatrist and hypnotherapy.

Both gastric acid and pepsin play a role as causative agents of the symptoms experienced with these conditions. The major component of gastric refluxate is pepsin, first discovered by Theodor Schwann (1836)[14] and is now considered to be the most aggressive proteolytic enzyme in the stomach.[15] Pepsin is secreted and synthesized as the

precursor pepsinogen by the chief cells located within the gastric mucosa.[16] When both pepsinogen and hydrochloric acid are present in gastric juice, pepsin takes its active form.[7] Once converted pepsin is activated and will continue to be aggressive in the absence of acid and is not deactivated until above pH 7.0.[17] Gastric juice in humans contains pepsin isoforms; 1, 3a, 3b, 3c; these isoforms have different characteristics, sensitivities, and "optimal pH level" when its action is at a maximum ensuring digestion occurs across a wide range of gastric pH.[18] The pepsin 3 complex is the most common state and this isoform contributes to 80% of human gastric juice.[19] Gastric juice comprises water, mucus, hydrochloric acid, pepsin, bile acids, and intrinsic factor. Of these 6 components of gastric juice, pepsin is the principal enzyme involved in the digestion of protein.[7] Literature on pepsin concentration levels within gastric juice is poor, a value of 0.9 ± 0.1 mg/mL has been reported.[20] Also pepsin concentration in gastric juice has been recorded as being between 0.5 and 1.0 mg/mL.[21]

Pepsin is an important component of gastric juice and present 100% of the time. Pepsin's importance is not just because of its powerful digestive enzymatic profile but also because it is a biomarker that identifies the presence of reflux disease in clinical samples. Its presence and concentration might be important to the diagnosis of GERD and identification of the different phenotypes of GERD as well as the possible role in the pathogenesis of phenotypes.

The study set out to evaluate the phenotypes of GERD, to demonstrate the relationship between gastric pepsin and gastric pH and that high gastric pepsin concentrations are associated with low gastric pH.

MATERIALS AND METHODS

Patient and Healthy Participants' Recruitment

Ninety patients were recruited from the GERD outpatient clinic, Section of Gastroenterology, Ege University School of Medicine, Izmir, Turkey. These included 52 patients diagnosed with ERD, 19 with NERD, 8 with EH, and 11 with FH. Twenty-four healthy controls (HC) were also admitted into the study recruited by Ege University School of Medicine. All the patients underwent high-resolution esophageal manometry (HRM) (MMS—Laborie, the Netherlands) and a multichannel intraluminal impedance-pH (MII-pH catheter; MMS-Laborie, the Netherlands). Acid exposure time (AET), baseline impedance (BI), symptom association probability (SAP), and symptom index (SI) of the participants were calculated. An AET value of < 4% as normal GERD and AET > 6% as pathologic GERD were accepted. BI values were taken at the sleeping period at night where reflux and swallowing did not occur. All patients were asked to abstain from eating for at least 6 hours before the procedure. PPIs, H2 receptor antagonists, and non-steroids were stopped 7 days and alginates and antacids were stopped 1 day before the procedure. HRM was performed to evaluate the upper limit of the LES and the peristalsis of the esophagus with a solid state 36-channel motility catheter. Following HRM, an MII-pH catheter was placed at 5 cm above the upper margin of the LES.

All HCs had upper gastrointestinal endoscopy to obtain the gastric juice sample and all controls had a negative history of upper gastrointestinal disease. Not all but most of the HCs had 24-hour pH impedance monitoring.

The exclusion criteria for the control group were primary esophageal motility disorders, Barrett's esophagus, cancer, chronic renal disorders, severe coronary artery disease, chronic obstructive pulmonary disease, upper gastrointestinal surgery, atrophic gastritis proven with biopsies, and severe comorbidity which may affect the study (Fig. 1).

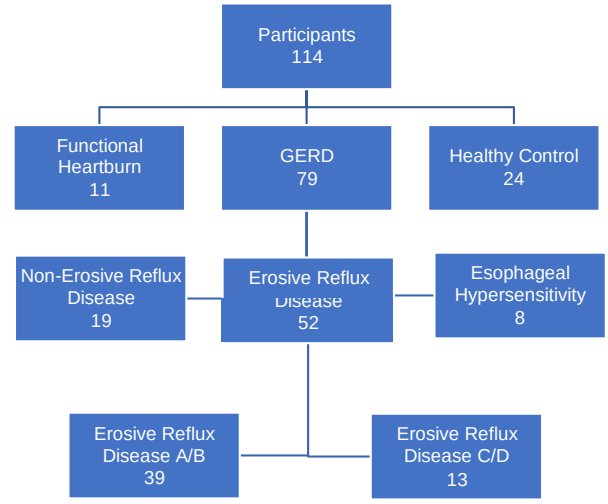

FIGURE 1. Patients with gastroesophageal reflux disease (GERD), functional heartburn, and healthy controls admitted into the study. full color online

Gastric Sample Preparation

Aspiration channels of the endoscopies were dried before endoscopy and the endoscopies advanced without suction. Gastric juice samples were aspirated at the beginning of upper gastrointestinal endoscopy into centrifuge tubes and placed immediately in a −20°C freezer. All upper gastrointestinal endoscopies and the collection of the gastric juice were performed under conscious sedation by the same physician (S.B.) at the Ege University Faculty of Medicine.

Peptest Analysis

Gastric juice samples were centrifuged for 5 minutes at 4000 rpm until a clear supernatant layer was visible. Gastric juice samples were then diluted (1:1250) with Migration Buffer (pH 8.2). From the diluted sample 80 µL was drawn up using an automatic pipette. The 80 µL sample was transferred to a microtube containing 240 µL of Migration Buffer. The 320 µL mixture was vortexed for 10 seconds. Using a pipette, 80 µL of the mixture was transferred to the circular well of a lateral flow device (LFD) containing 2 unique human monoclonal antibodies; 1 to detect and 1 to capture any pepsin present in the clinical sample (Peptest, RD Biomed Limited, UK). A "control" line was produced within the window of the LFD if the test was successful, and a "test" line was generated if the sample contained pepsin. The intensity of the "test" line was measured using an LFDR101 reader; this measurement was used to calculate the concentration of pepsin within the gastric juice sample in mg/mL (Fig. 2).

Gastric Sample pH Measurement

The pH measurement of the majority of the gastric juice samples (n = 104) was taken and recorded immediately on arrival into the laboratory (predilution) using Hanna Instrument pH Meter, HI 8424.

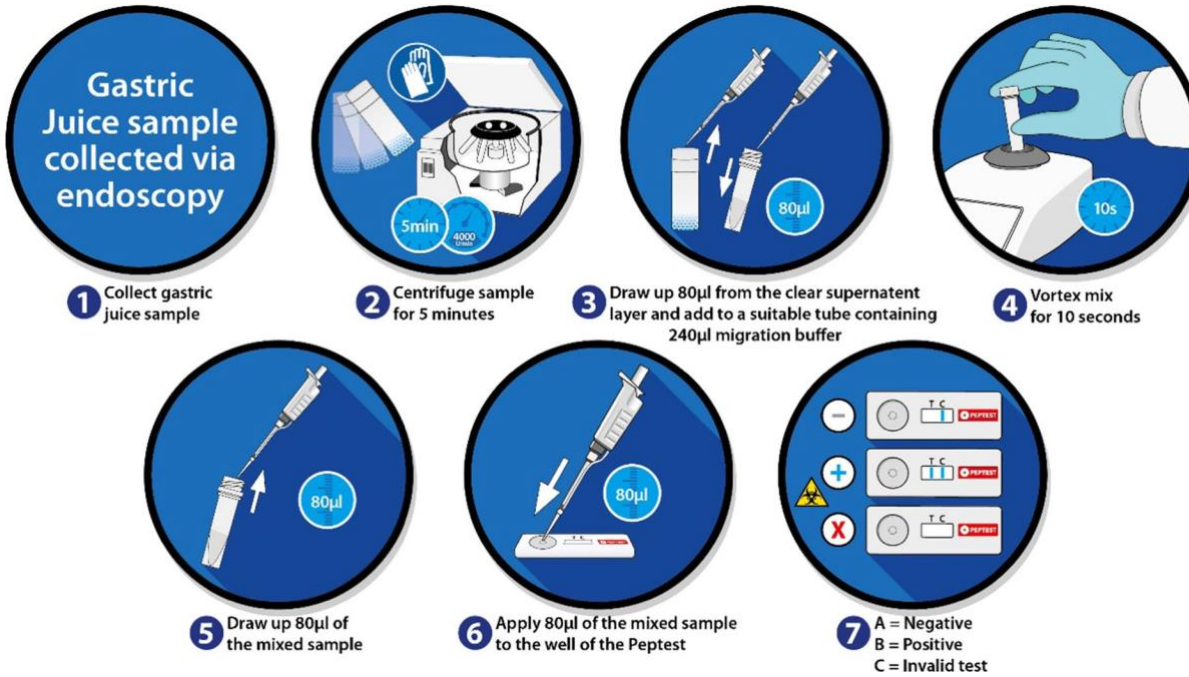

FIGURE 2. Schematic process for the collection and analysis of gastric juice samples to use Peptest to determine pepsin concentration within the clinical sample. full color online

Statistical Analysis

Data were analyzed using the statistical package GraphPad Prism 9.0.1 (GraphPad Software, San Diego, CA). Data were expressed as mean ± SD for variables with normal distribution and median (interquartile range) for variables with non-normal distribution. Multiple group comparisons were performed using 1-way analysis of variance followed by Tukey test for normal distributed data and the Kruskal-Wallis test with Dunn comparison for non-normal data. The correlation between gastric pepsin and gastric pH was demonstrated using linear regression. *P* values <0.05 were statistically significant.

RESULTS

A total of 90 patients and 24 HCs were recruited to take part in the study. Ethical approval was obtained, and gastric juice samples were aspirated (5 mL minimum volume) during upper gastrointestinal endoscopy. The 90 patients were recruited from 5 previously diagnosed clinical groups as shown in Table 1 according to upper gastrointestinal endoscopy, 24-hour pH impedance, and esophageal high-resolution manometry. The largest groups were those diagnosed with ERD A/B. The mean age of the patients and the HCs were similar for each group except for those patients presenting with ERD C/D. In the patient group, there were 52 males and

TABLE 1. Demographical Characterization of Healthy Controls and Patient Subgroups (n = 114), Gastric Pepsin (Mean ± SD), and Gastric pH (Median) Values for Each Group

	Healthy Controls	ERD A/B	ERD C/D	NERD	Esophageal Hypersensitivity	Functional Heartburn	Total
n	24	39	13	19	8	11	114
Age, mean ± SEM (y)	42 ± 2.8	41 ± 2.0	51 ± 2.9	41 ± 3.4	40 ± 3.3	39 ± 3.5	
Male/female	7/17	26/13	8/5	9/10	3/5	6/5	59/55
BMI	23	28	27	25	24	25	
Smokers (yes/no)	7/13	13/25	2/11	4/15	0/8	3/8	29/80
Alcohol (yes/no)	7/13	12/27	7/6	3/16	3/5	0/11	32/78
Pepsin (mg/mL)							
Mean	0.750	0.705	0.865	0.576	0.813	0.788	
SD	0.467	0.373	0.410	0.348	0.501	0.359	
Minimum	0.028	0.066	0.183	0.016	0.297	0.295	
Maximum	1.951	1.633	1.501	1.121	1.474	1.266	
pH							
Median	1.9	1.6	1.4	2.5	1.5	1.6	
Minimum	1.1	0.6	1.1	1.3	1.1	1.2	
Maximum	7.1	6.6	3.4	6.0	3.1	1.8	

BMI indicates body mass index; ERD, erosive reflux disease; NERD, nonerosive reflux disease.

TABLE 2. Patient Group Details for AET, SI, SAP, and BI

	AET	SI	SAP	BI (Ω)
ERD A/B	9.9 ± 5.5	62.4 ± 28.9	91.4 ± 13.3	1100.9 ± 718.3
ERD C/D	23.9 ± 25.2	62.6 ± 24.2	91.9 ± 12.6	433.7 ± 200.8
NERD	7.5 ± 3.4	69.5 ± 24.6	96.4 ± 8.7	1563.3 ± 720.3
EH	1.7 ± 1.3	63.8 ± 23.1	98.7 ± 2.1	2285.1 ± 612.7
FH	1.5 ± 1.0	27.5 ± 13.6	87.3 ± 6.3	2117.9 ± 585.7
HC	1.4 ± 2.0	NA	NA	2282.4 ± 610.9

AET indicates acid exposure time; BI, baseline impedance; EH, esophageal hypersensitivity; ERD, erosive reflux disease; FH, functional heartburn; HC, healthy control; NA, not applicable; NERD, nonerosive reflux disease; SAP, symptom association probability; SI, symptom index.

38 females. In the HC group, there were 17 females and 7 males. The mean BMI (normal to overweight) was similar across all groups and smoking and alcohol consumption was generally low across all the groups.

The mean± SD gastric pepsin levels (mg/mL pepsin) across all patient groups and HCs are shown in Table 1. The highest pepsin concentration was observed in the patients diagnosed with ERD C/D (mean 0.865 mg/mL with a range: 0.183 to 1.501 mg/mL) and this group had a higher level of gastric pepsin compared with the NERD patient group (mean 0.576 mg/mL with a range: 0.016 to 1.121 mg/mL) and was not significantly different from all other patient groups and the HC group (mean 0.750 mg/mL with a range: 0.028 to 1.951 mg/mL).

The diagnostic criteria of each patient group entered into the study are shown in Table 2.

ERD A/B and ERD C/D patients had pathologic upper GI endoscopy and AET > 6% on 24-hour esophageal pH monitoring. ERD C/D had the lowest BI values versus other groups. The upper gastrointestinal endoscopy findings of NERD, EH, and FH were normal. NERD and EH patients had positive SAP and SI. AET of NERD was > 6%, while EH was <4% on 24-hour esophageal pH monitoring. However, FH patients had an AET <4% and negative SI, SAP.

Gastric samples had pH measured before pepsin analysis; this was an important aspect of the study comparison between groups of pH and pepsin concentration. The median gastric pH values for all groups are shown in Table 1. The following significant differences were calculated between groups. The gastric pH of the ERD A/B (median 1.6, range: 0.6 to 6.6) group was significantly lower (P = 0.0018) when compared with the NERD group (median 2.5, range: 1.3 to 6.0) and lower when compared with the HC group (median 1.9, range: 1.1 to 7.1). The gastric pH of the ERD C/D (median 1.4, range: 1.1 to 3.4) group was significantly lower (P = 0.0039) when compared with the NERD group. The pH of the EH (median 1.5, range: 1.1 to 3.1) group was significantly lower (P = 0.0233) when compared with the NERD group. The gastric pH of the FH (median 1.6, range: 1.2 to 1.8) group was significantly lower (P = 0.0042) when compared with the NERD group. Table 3 shows the P values obtained following analysis of variance analysis for each group comparison for both gastric pepsin and for gastric pH.

There was a correlation between pepsin concentration and the pH of gastric juice samples. The ERD C/D group presented with the highest mean pepsin concentration (0.865 mg/mL) and the lowest median pH (1.4). The NERD group had the lowest mean pepsin concentration (0.576 mg/mL) and the highest median pH (2.5).

The correlation between gastric pepsin and gastric pH was further demonstrated using regression analysis for individual participants in each study group and plotting the values for gastric pepsin against the values for gastric pH. This resulted in a significant (P = 0.0117) linear relationship between the gastric pepsin and the gastric pH group values as shown in Figure 3.

DISCUSSION

Pepsin has a long history since first its discovery in 1836 by Theodor Schwann[14] and present in all vertebrates such as fishes and mammals.[22] The stomach is largely devoid of live organisms which is because of the presence of gastric acid and also because of acid activating pepsinogen, which releases pepsin and in turn plays a vital role in digestion through proteolysis, further helping to keep the stomach free of bacteria.[23] Both pepsin and pH play a major role in the bactericidal activity in the stomach, a traditional view was that pepsin was most active at pH 2 to 3 with activity declining as the acidity diminished.[24]

It was always assumed that pepsin would be destroyed or rendered inactive by high-dose PPIs and there was skepticism that pepsin played a role in damaging extraesophageal tissues but the last few years of basic and clinical research has changed this perception.[8] The activity and stability of pepsin is very closely related to the prevailing pH and it is important when investigating pepsin activity to record the pH of the

TABLE 3. Analysis of Variance Analysis Across the Healthy Control Group and All Patient's Subgroups Showing All *P* Values for the Gastric Pepsin and Gastric pH Analysis

	ERD C/D	NERD	Esophageal Hypersensitivity	Functional Heartburn	Healthy Controls
Gastric pepsin					
ERD A/B	0.8190	0.8616	0.9829	0.9906	0.9980
ERD C/D		0.3560	0.9997	0.9973	0.9628
NERD			0.7305	0.7333	0.7223
Esophageal hypersensitivity				> 0.9999	0.9989
Functional heartburn					0.9998
Gastric pH					
ERD A/B	> 0.9999	0.0018	> 0.9999	> 0.9999	0.2056
ERD C/D		0.0039	> 0.9999	> 0.9999	0.1560
NERD			0.0233	0.0042	> 0.9999
Esophageal hypersensitivity				> 0.9999	0.3843
Functional heartburn					0.1133

ERD indicates erosive reflux disease; NERD, nonerosive reflux disease.

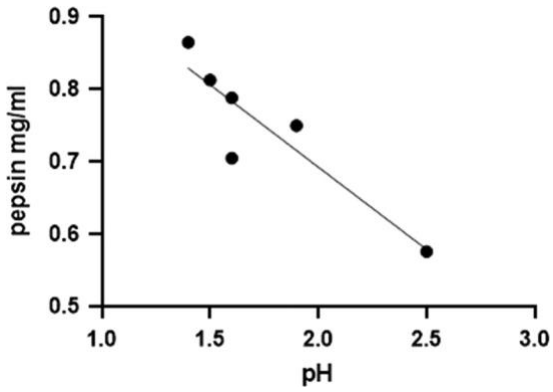

FIGURE 3. Regression analysis for individual participants in each group demonstrating the significant linear relationship between the mean gastric pepsin against the median gastric pH (P= 0.0117).

gastric environment. The correlation between pepsin concentration and the pH for gastric juice samples in each GERD study group and the HC group is illustrated well in Table 1 in the results section. Both the pepsin and the pH followed the severity of erosive esophagitis. ERD C/D present with the highest mean intragastric pepsin concentration (0.865 mg/mL) and the lowest median gastric pH (1.4). The NERD group had the lowest mean gastric pepsin concentration (0.576 mg/mL) and the highest median gastric pH (2.5).

Individual pepsin isoenzymes were shown to be stable for 24 hours at body temperature but were ultimately degraded by autocatalysis if stored at its optimal pH.[25] However, this contrasted with mixtures of pepsin isoenzymes as found in gastric juice, like the gastric samples investigated and reported in the current study were shown to be far more stable. This led to the finding that purified porcine pepsin was irreversibly denatured at pH 7.1, whereas peptic activity of human gastric juice persisted until exposed to pH 7.8 because of the presence of pepsin.[26]

In the perfect digestive tract pepsin remains in the stomach and is active within the stomach as the enzyme responsible for digestion and bactericidal activity. However, research has demonstrated how gastric juice and therefore pepsin travel retrogradely up the esophagus and can continue to travel into the larynx and the airways.[27] Studies have demonstrated pepsin to be a biomarker of reflux and that it is associated with laryngopharyngeal reflux, various respiratory diseases, lung disease, asthma, chronic cough, dysphonia, hoarseness, sore throat, throat clearing, and other ENT-related diseases.[28] The source of the pepsin found in the airways and in saliva is gastric refluxate and one of the main aims of the present study was to investigate gastric pepsin concentration and the gastric pH in patients diagnosed with different GERD phenotypes and FH in comparison with HCs. We want to evaluate the role of gastric pepsin in the pathogenesis of different GERD phenotypes. We believe that this is the first study to investigate pepsin concentration and pH profile in different reflux phenotypes. In the literature, we found little information on basal gastric pepsin levels and no studies on gastric pepsin levels in diagnosed GERD and FH patients. In a study published in 1988 by Ten Kate et al[20] using healthy male volunteers (n = 8) the basal pepsin concentration quoted was 0.9 ± 0.1 (0.5 to 1.5) mg/mL in comparison with HCs (n = 24); the current study had a basal pepsin concentration of 0.8 ± 0.1 (0.03 to 1.95) mg/mL. An interesting observation reported in the Ten Kate study was that despite a marked profound decrease in gastric acid secretion following the PPI omeprazole there was no effect on gastric pepsin secretion in healthy volunteers. Ten Kate et al[20] concluded that acid and pepsin secretion are at least partly governed by independent mechanisms. Gastric pepsins may also contribute to gastroduodenal ulceration. Previously the perception had been that PPI treatment renders pepsin inactive by elevating the gastric pH, this is now an outdated view especially as we now know that pepsin retains activity up to pH 7.8 and be reactivated when exposed to acid and can damage cells even in the absence of acid.[19]

When reviewing different phenotypes, it is interesting to divide reflux into 2 main groups. The first one an erosive group related to esophageal damage as a consequent of the presence of acid and pepsin. The second a nonerosive group related more to hypersensitivity and not to damage caused by acid and pepsin. In the present study, the patient group with the highest pepsin level were those patients diagnosed with ERD LA grade C and D with a mean gastric pepsin level of 0.865 mg/mL (0.183 to 1.501 mg/mL), which was higher when compared with patients diagnosed with NERD mean gastric pepsin level of 0.576 mg/mL (0.016 to 1.121 mg/mL). This observation fits well as the ERD group will be associated with the most esophageal epithelia damage and the greater erosion so could be predicted to have the highest level of gastric pepsin. The pepsin levels in other patient groups were not significantly different from those found in the HC group with a mean pepsin level of 0.750 mg/mL (0.028 to 1.951 mg/mL). Our results support the theory that pathogenesis of EH and FH might be independent of the noxious effect of pepsin.

All the gastric samples had pH measured before pepsin analysis and the median pH values for all groups were compared. An interesting picture emerged as the patient group with the lowest median gastric pH 1.4 (1.1 to 3.4) was the group diagnosed with ERD LA grades C and D, which was significantly (P = 0.0039) lower than the median gastric pH of the NERD patient group pH 2.5 (1.3 to 6.0). The gastric pH of the NERD patient group was higher than that recorded for the HC group. The median gastric pH of the other 3 patient groups was similar and all significantly lower than the NERD group. The median gastric pH profiles fitted well with what would be predicted, the lowest medium gastric pH observed with the patient group ERD LA grade C and D presenting with the most esophageal epithelia damage.

The main limitation of the study was the low patient numbers in some of the GERD phenotype groups especially in patients diagnosed with EH and FH. Higher patient numbers would have improved the comparative group analysis.

Overall, there was good correlation and a significant linear relationship between gastric pepsin levels and gastric pH within the patient groups demonstrating just how sensitive these measurements are and showed that the GERD phenotype (ERD C/D) with maximum erosion had the highest gastric pepsin concentration and the lowest gastric pH.

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